BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

Woods Hole, Mass.
Biological bulletin

WOODS HOLE, MASS.

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Gen. Walker

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BIOLOGICAL BULLETIN

THE FERTILIZATION-REACTION IN *ECHINARACHNIUS PARMA*. VI.

The Necessity of the Egg Cortex for Fertilization.

E. E. JUST,[1]

Rosenwald Fellow in Biology, National Research Council.

If we define fertilization as an instantaneous irreversible reaction at or in the cortex of the egg between an ovogenous substance, fertilizin, and the spermatozoön, it must follow (1) that an egg, once fertilized, is incapable of response to additional insemination, and (2) that fragments of fertilized eggs are likewise incapable of fertilization. If, moreover, the fertilization-reaction be limited to the cortex, then it must likewise be shown (3) that uninseminated eggs, or fragments thereof, devoid of cortex are not fertilizable. The present paper aims to set forth certain observations made at the Marine Biological Laboratory, Woods Hole, Mass., on the egg of *Echinarachnius parma* which indicate that fertilized eggs, or fragments thereof, are unfertilizable; and that uninseminated eggs, or fragments thereof, devoid of cortex are likewise unfertilizable. It is therefore concluded that the cortex of the uninseminated egg is necessary for fertilization.

II.

The fertilized egg does not react to additional insemination; sperm do not enter fertilized eggs. In order to test the validity of this generally accepted statement, fertilized eggs after removal of their membranes have been inseminated two, three, and four minutes after insemination and at later stages during development to gastrulation. Such eggs have been sectioned.

In no case have sperm been found in the egg or blastomeres after even the heaviest insemination. Rupture of the blastomeres

[1] Zoölogical Laboratory, Howard University.

with outflow of cytoplasm does not facilitate sperm entry. To offset the possibility that fixation might be a source of error, most diverse fluids were used. In the living egg, in addition, it was easy to see that spermatozoa do not react with fertilized eggs. There is here, certainly, no evidence in support of Kohlbrugge's results.

Many experiments have likewise been made thus: Eggs are lightly inseminated and at five-second intervals up to the time of membrane separation are given an additional heavy insemination. Such eggs fail to reveal polyspermy in higher per cent. than eggs that have but one insemination.

Thus, June 28, 1918, eggs of *Echinarachnius* were inseminated and at five-second intervals up to time of membrane separation were reinseminated. After membrane separation, the eggs were gently shaken to remove the membranes; samples of these were reinseminated at six, thirteen, sixteen, and twenty minutes after the original insemination. Samples of these eggs were fixed in corrosive sublimate-acetic two minutes after each insemination. No evidence of a reaction was found in any of the sectioned material.

In addition, the bulk of the evidence (Lillie, '19) shows that artificially activated eggs fail to react with sperm.

Wilson found that fragments of fertilized eggs of *Cerebratulus* are incapable of fertilization. Conklin has shown that the unusually large polar bodies produced by the egg of *Crepidula* through centrifugal force do not fertilize. Since in this egg polar-body formation follows insemination, Conklin's results are capable of the interpretation that here, too, parts of fertilized eggs do not refertilize.

In *Echinarachnius* the situation is the same. If inseminated eggs of *Echinarachnius* be gently shaken in a vial with bits of broken coverslips and the fragments thus obtained be divided into two lots, one of which is inseminated, the per cent. of development in the two lots is the same. The insemination of these fragments, even if made *twenty seconds* after the insemination of the intact eggs, does not increase the per cent. of development.

These observations indicate that fertilization is irreversible, eggs

completely activated can not respond to additional insemination; and fragments of inseminated eggs behave similarly.

Through the well-known experiments of the Hertwigs, Boveri, Morgan, and others it has been shown that enucleated fragments from uninseminated eggs of sea-urchins are fertilizable as are nucleated fragments. Wilson has shown that the enucleated fragments taken from the egg of *Cerebratulus* after dissolution of the germinal vesicle are fertilizable. In a very cautiously worded paper appearing posthumously Boveri maintained his original position as to the fertilizability of enucleated fragments of uninseminated eggs.

I find that fragments from uninseminated eggs of *Echinarachnius* obtained by gently shaking the eggs in a vial with bits of a broken coverslip are capable of fertilization and development. The development of these fragments does not depend upon the presence of the egg nucleus. Some fragments without egg nuclei fail to respond to insemination. A fragment of large size and with a nucleus may not fertilize. Very small fragments with no egg nucleus develop. *I believe that the failure of fragments to fertilize is due to the absence of cortical material.* This belief is based on results which may now be considered.

III.

Toward the end of the season of 1917 I frequently found that fertilized eggs of the *Echinarachnius* gave rise to abnormal gastrulæ which I took to be ordinary exogastrulæ. They prove to be gastrulæ with masses of undifferentiated protoplasm attached.[1] The breaking up of these masses simulates cleavage. A careful study of these eggs was made and the history of this condition revealed.

I found that among various lots of eggs kept for some time in shallow dishes with little sea-water were some eggs which on return to a larger quantity of normal sea-water underwent a fragmentation. Under the microscope this process is easily followed. The eggs give off a bud or form an exovate that slowly increases in size and drops off. Thus in a given lot of eggs there are in-

[1] These masses are always located at the vegetative pole. This may be significant for the problem of polarity.

numerable cases each with a bud of varying size still attached to or just detached from the egg. If insemination takes place before the bud drops off, a membrane separates from the "egg" and never from the bud. Repeated observation puts this statement beyond doubt. I have never seen two membranes on such eggs, nor a single membrane enclosing both egg and bud. The portion within the membrane alone cleaves and develops. The portion outside the membrane never develops; it remains attached to the gastrula until completely disintegrated. In some cases the bud is so much larger than the "egg" that membrane separation takes place from a relatively small disk; the cleavage of such eggs is discoidal; such eggs never give rise to swimmers.

If the observer inseminate eggs after the buds drop off, only one member of a pair separates a membrane, cleaves, and gastrulates, though it may be the smaller. The presence or absence of the egg nucleus is of no consequence for the development of these fragments.

Any one of three possibilities was thought of as responsible for this phenomenon of bud formation in the egg of *Echinarachnius:* (1) staleness of the eggs, (2) the presence of blood, (3) the general deterioration of the sexual products toward the end of the season. Accordingly, in 1918 attempts were made to ascertain which of these factors is responsible for this bud formation. And we may state at the outset that though each factor may contribute to the production of buds, *the essential factor is the hypertonicity of the medium.*

If eggs of *Echinarachnius* are allowed to stand in sea-water for several hours, they slowly undergo changes that eventually lead to their complete disintegration. A portion of these eggs upon insemination separate membranes many of which are stuck to the swollen cortex. If previous to insemination these eggs be gently shaken, buds are formed from those with swollen cortex. Such inseminated eggs with buds separate membranes only from the "eggs" and never from the buds. These eggs cleave and gastrulate, but the per cent. is always low. Late in the season buds are more easily produced. And throughout the season the presence of blood increases the number of buds formed.

By far the easiest method for the production of a high per cent.

of buds at any time during the season is to allow the uninseminated eggs to stand in a small quantity of sea-water in a shallow dish, thus permitting evaporation; or, better, to place uninseminated eggs in hypertonic sea-water (6 parts of 2½ *M* NaCl plus 50 parts sea-water). On transfer of the eggs to normal sea-water they are gently shaken or squirted through a pipette. Large numbers of such eggs produce buds.

On insemination membranes separate from but one component of these budded eggs. Only that portion of the egg within the membrane divides and gastrulates. The gastrulæ swim attached to the undifferentiated mass of budded cytoplasm which eventually disintegrates. The process of bud formation is easily followed under the microscope and insemination made at any stage. Insemination made after complete separation of the bud gives the same result: in any two given masses of egg material separated by constriction of a bud one only develops, regardless of size or the presence of the egg nucleus.

The explanation of these results on budded eggs of *Echinarachnius* is as follows: The cortex of the eggs changes under various forms of treatment. As the uninseminated egg of *Echinarachnius* lies in sea-water it slowly deteriorates. A distinguishing mark of this deterioration is the physical change in the cortex: the cortex is thick and practically transparent. Late in the season also many eggs are found with thick cortices. Blood, too, will frequently hasten this change in the cortex. Now, hypertonic sea-water very clearly brings about a physical change in the cortex. After exposure to hypertonic sea-water the cortex may be readily seen as a thick jelly-like hull enclosing the egg. It is from this jellied cortex that the membrane separates on insemination.

If an egg with a thick cortex be gently shaken on transferal to normal sea-water, the cortex breaks and the contents of the egg flows out. Indeed, merely the transfer from hypertonic sea-water to normal sea-water will tend to produce this outflow in some eggs, as they rapidly take up water. The bud is thus made up of endoplasm and is without cortical material. In favorable cases this is readily determined.

And only that component of the budded egg which has the clear rim of cortex is fertilized on insemination as revealed by the pres-

ence of the membrane, cleavage, and gastrulation. The naked mass of endoplasm rounds up still attached to the developing egg. It never reacts with sperm whether inseminated while attached to the egg or after separated from the egg. Thus the presence of the egg cortex is necessary for fertilization. Many observations make this interpretation certain.

In the egg of *Arbacia* the results are the same; indeed, if anything, they are more clear-cut.

Hypertonic sea-water is not the only agent that will bring about this outflow of endoplasm. Frequently shaking will bring it about in a few eggs of a given lot. Hypertonic sea-water is best, however. first because it produces a high per cent. of budded eggs, and second because it makes very clear that the cortex is on the egg and not on the endoplasmic mass.

One additional method may be mentioned now because its use has in turn led to some interesting experiments along another line. This method involves the use of bolting silk, soft filter paper, and lens paper. We may briefly consider this method.

Uninseminated eggs of *Echinarachnius* are dropped on bolting silk (in focus under low power of the microscope), the mesh of which has a diameter less than that of the egg, stretched above the surface of sea-water in a stender dish. If the concentration of eggs in the drop of sea-water is just right, some eggs rupture as they flow through the meshes of the silk. If the observer work rapidly, he can after trial inseminate these eggs just as they burst. The silk is then quickly thrust into the dish of sea-water. Some of the eggs form membranes with naked buds attached.

With filter paper the method is much the same. Soft moist filter paper on which is placed a drop of eggs is mounted under the microscope above sea-water in a low stender dish. The eggs flow beneath the fibers of the filter paper and thus burst because of pressure and slight drying. As they burst they are inseminated and the paper plunged into sea-water. Some of these eggs later show buds without membranes attached to cleaving eggs within membranes. Intact eggs inseminated among the fibers of filter paper in sea-water on insemination will develop normally. I have kept such eggs through to the pluteus stage. With lens paper one may obtain much the same results; the lens paper, in addition, is

much easier to handle: the endoplasmic outflow is more readily followed.

With a little care one may induce flow of endoplasm through the cortex. The naked endoplasm rounds up and in appearance is like the remaining part of the egg. But the endoplasm does not fertilize; it fails to react with sperm.

Here, again, eggs of *Arbacia* give comparable results.

While my observations were under way in 1918, Dr. Robert Chambers informed me that by the method of microdissection he was able to remove the cortex from the egg of the starfish (Lillie, '19). Such eggs are incapable of fertilization. Portions of the egg with cortical material, on the other hand, readily fertilize.

We may conclude from these observations that certain forms of treatment so alter the cortex as to facilitate endoplasmic outflow. By such treatment the fertilization capacity of the egg is not lost; it is, however, localized in only that part of the egg enclosed by cortical material. It thus follows that the inner substance of the egg is non-fertilizable in fertilized eggs not because of progressive centripetal changes set up at the cortex on insemination, but because the endoplasm is inherently non-fertilizable. Again, it is not necessary to postulate that the development of fragments from uninseminated eggs following fertilization may be due to the presence of some nuclear material of the egg (cf. Boveri). If the interpretation of the observations here reported be correct, fragments of uninseminated eggs, whether nucleated or not, are fertilizable if they possess cortical material. The egg cortex is thus necessary for the fertilization-reaction.

IV.

In any attempt at defining fertilization we must consider several facts.

In the first place, animal ova vary with respect to the stage in their development in which they are fertilized. Thus some reach the fertilizable condition before the germinal vesicle breaks down, others in the mesophase of the first maturation, still others during the second maturation, and many after maturation is complete. Starfish eggs may be fertilized at any time from the dissolution of the germinal vesicle to a short time after complete maturation.

Nor, again, is mere sperm penetration fertilization, since sperm normally penetrate ova (*Dinophilus, Saccocirrus,* etc.) some time before fertilization ensues. There are thus all possible types of fertilization with respect to the maturation stage of the egg when normally inseminated. No definition of fertilization is worth while if based on one type of egg alone.

In the second place, though the end result of fertilization is cleavage, there are here, too, many differences among animal ova. Thus the zygote nucleus may at first divide without cytoplasmic division (*Renilla*); the germ nuclei may fuse or appose merely; the cleavage spindle may be homodynamic, or heterodynamic; the sperm amphiaster may be homo- or heterodynamic, its second aster arising before or after union with the egg nucleus; and the cleavage centers may arise about the sperm nucleus or the egg nucleus or in part about each. A definition of fertilization in terms of the behavior of the germ nuclei or of the origin of the cleavage centers is manifestly inadequate.

If, for example, we consider the classic theory of Boveri that fertilization is due to the introduction of centrosomes by the spermatozoön, we realize its inadequacy at once, since it demands that the middle-piece enter the egg. It is true that the whole spermatozoön enters certain eggs whose maturation spindles are without centrosomes or asters, thus apparently supplying a deficiency. But in many other cases the middle-piece does not enter the egg, and where it does as in echinid ova the identity of its so-called centrosomes is wholly mistaken. To support the Boveri hypothesis we must shift the position of the potent centrosomes to fit those cases where the middle-piece does not enter the egg, or on entering takes no part in aster formation.

Because of failure to recall these simple facts purely morphological theories of fertilization fail. Indeed, many studies on fertilization are but studies of cell division; they deal with structures and phenomena in cell division in no wise restricted to egg cells. Nor yet have many physical or chemical theories been more fortunate. These theories are based on the study of physiological changes incident to cell division. But cell division is not fertilization.

An approach to the fertilization problem can be made only

through study of fertilization in the most diverse types of ova and by rejection of the incidental phenomena for the basic and common. What is the common factor in fertilization? So far as we know, it is some type of cortical change. But by cortical change we do not mean that the sign of the thing is the thing itself. Thus membrane separation in the sea-urchin egg is an easily visible sign of cortical change. Membrane separation, however, is not fertilization. It is here that an error lies in much of the work on experimental parthenogenesis.

Though we may doubtless gain knowledge of the nature of the cortical changes following insemination through study of these changes experimentally induced, we can not rely wholly on such work to explain fertilization. A far more simple mode of attack is to study fertilization itself. And if, in addition, the theory of the action of the agent in experimental parthenogenesis is erroneous, such a theory for fertilization can but hinder the solution of our problem. If it be true that cell division is not fertilization, it is equally true that experimental parthenogenesis is not fertilization. We must, therefore, study the fertilization process itself, the common factor of which is some kind of cortical change.

The evidence at hand indicates that the cortical changes in fertilization are due to an instantaneous, irreversible reaction between an ovogenous substance, fertilizin, and the spermatozoön. Stated in these terms the theory almost demands that the cortex is necessary for fertilization. The evidence herewith submitted points to this conclusion. The primary, if not, indeed, the whole event in fertilization, is the cortical reaction. The succeeding events with concomitant physical and chemical changes leading to cell division and development are the consequence of a complete cortical reaction between fertilizin and spermatozoön.

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THE FERTILIZATION-REACTION IN *ECHINARACHNIUS PARMA*. VII.

The Inhibitory Action of Blood.

E. E. JUST,[1]

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The present communication aims to set forth results of experiments made during two seasons (1919 and 1920) at the Marine Biological Laboratory, Woods Hole, Mass., to test that part of Lillie's fertilizin theory which postulates that blood (in *Arbacia*) inhibits fertilization through intervention of the fertilizin and the egg (Lillie, '14). The present writer was firmly of the opinion that this postulated action might be merely a surface effect: that despite the agglutination of *Arbacia* sperm, by *Arbacia* egg-water in the presence of specific blood, the main action of the blood is on the surface of the egg so that sperm can not enter. The results of the experiments here reported, however, show that, in the egg of *Echinarachnius parma* at least, this is not the case: blood blocks fertilization in this egg by interfering with the reaction of fertilizin and egg and not with the sperm and fertilizin at the surface of the egg. For repeated observations reveal that both in straight and cross fertilization, with *Arbacia* sperm, eggs of *Echinarachnius* inseminated in blood, though they fail to develop, nevertheless take in sperm. We may divide the experiments into two groups: those that deal with straight fertilization and those that deal with cross fertilization with *Arbacia* sperm.

I.

Eggs of *Echinarachnius* obtained by cutting up ovaries in sea-water invariably give low fertilization percentages. Thus the early observations—1910, 1914, 1915—made on such eggs gave the impression that this is a poor egg for the study of fertilization. An egg suspension strained from ovaries cut up in sea-water shows a slight turbidity or greater depth in color depending upon the amount of blood and detritus present. My notes indicate that fertilizing power falls off with increasing depth of color. With shed eggs, on the other hand, the case is quite different: they invariably yield 100 per cent. fertilization. If, however, shed eggs

[1] Zoölogical Laboratory, Howard University.

of maximum fertilization capacity be inseminated in cœlomic fluid, the per cent. of cleavage is decreased. Thus equal parts of cœlomic fluid and sea-water may cut down the per cent. of cleavage to zero; higher proportions of cœlomic fluid, 75 to 100 per cent., invariably permit no fertilization.

In practice it was found extremely difficult to use large quantities of blood owing to its scarcity. Since, however, eggs from one female only were used in any given experiment, this was found no great difficulty, since the number of eggs used was very small in each case.

The method used is about as follows: Equal parts of cœlomic fluid and sea-water made solution No. 1. To half of No. 1 was added a like quantity of sea-water to make No. 2. Thus a series of half dilutions was made. One half of the last member in the series was discarded in order that all numbers would contain the same quantity of solution. Uninseminated eggs were placed in each solution—one drop of an egg suspension to each. Likewise a drop of uninseminated eggs was placed in normal sea-water equal in amount to that of mixture of cœlomic fluid and sea-water. The eggs in all dishes were then inseminated with the same amount of sperm from one male. In general, inseminations were made first in 100 per cent. and in 50 per cent. blood. Unless these gave high percentages of inhibition, I made no further dilutions.

The appended summary (Table I.) gives the results of six experiments made in 1919:

TABLE I.

THE INHIBITORY EFFECT OF SPECIFIC BLOOD ON FERTILIZATION OF THE EGG OF *Echinarachnius parma* AS REVEALED BY THE PER CENT. OF CLEAVAGE IN VARIOUS CONCENTRATIONS OF BLOOD IN SEA-WATER IN 6 EXPERIMENTS OF 1919.

No.	Per Cent. of Blood in Sea-water.	Per Cent. of Cleavage.					
		Exp. 1.	Exp. 2.	Exp. 3.	Exp. 4.	Exp. 5.	Exp. 6.
1.....	100	0	0	0	0	0	0
2.....	50	0	0	0	0	0	0
3.....	25	0	0	0	3	6	0
4.....	12.5	0	1	0	8	33	0
5.....	6.25	0	12	16	14	50	0
6.....	3.125	49	40	47	33	81	0
7.....	1.5625	89	64	78	90	90	0
8.....	0 (control)	99	100	98	98	99	36

It is thus seen that eggs of high fertilization capacity fail to fertilize if inseminated in the presence of certain concentrations of blood. Not all eggs give results comparable to those in the table. Thus during May, 1921, several samples of blood tested showed very weak inhibitory power. In essentials, however, the results are quite comparable to those obtained by Lillie in his study of fertilization in *Arbacia*. Moreover, Lillie's interpretation of the mode of action of the blood inhibitor is sustained by this work on the egg of *Echinarachnius*, as will now be shown.

Sperm of *Arbacia* readily agglutinate in mixtures of *Arbacia* egg-water and blood as though the blood were absent. Lillie thus concluded from this that the blood does not block the reaction between the sperm-agglutinating substance (fertilizin) and the sperm; the block comes between fertilizin and substances in the egg. But it is at once apparent to the reader that this is not wholly conclusive: the substance in blood that inhibits fertilization may well do so by some action on the surface of the egg rendering sperm attachment and penetration impossible. Thus it might well be that sperm in the presence of blood and egg-water rich with sperm agglutinin of high power agglutinate; but in ordinary insemination this amount of agglutinin is not present, nor is the insemination made as heavy as the sperm suspensions must be to detect the presence of agglutinin. In the inseminations usually employed for fertilizing eggs agglutination of spermatozoa does not occur; instead, the spermatozoa stick to the egg. As a matter of fact, sperm likewise stick to *Echinarachnius* eggs inseminated in blood. The failure of such eggs to fertilize can not, therefore, be attributed to the effect of blood in blocking the agglutination of sperm to the egg.

At first I considered this result as due to the poor quality of the sperm; that it was not so much an inhibition by blood as a failure of fertilization. Subsequently it was found repeatedly that on inseminating in the presence of blood spermatozoa are attached to the eggs. Thus we have evidence to support the postulate offered by Lillie as to the mode of action of blood inhibitor. This is brought out again in the next group of experiments.

II.

It has been shown (Just, '19) that the fertilization of eggs of *Echinarachnius* by *Arbacia* sperm is greatly facilitated by the use of alkali or by heavy insemination. Though giving a lower per cent. of cleavage than alkali, heavy insemination was for several reasons the method adopted in the experiments made to determine the effect of *Echinarachnius* blood on fertilization by *Arbacia* sperm. That this cross is inhibited by blood was suggested in the earlier work. The experiments now cited indicate that this is true. I cite four experiments made in June and in August, 1920.

Uninseminated eggs of *Echinarachnius* are washed in sea-water and allowed to settle. Five drops of this suspension is distributed equally among five dishes as follows: *Lot A,* uninseminated in sea-water; *Lot B,* inseminated in sea-water with *Echinarachnius* sperm; *Lot C,* inseminated in 3 per cent. *Echinarachnius* blood with *Echinarachnius* sperm; *Lot D,* heavily inseminated in 3 per cent. *Echinarachnius* blood with shed *Arbacia* sperm; *Lot E,* heavily inseminated in sea-water with shed *Arbacia* sperm. The results follow:

Lot.	Per Cent. of Cleavage.			
	June 22.	June 24.	August 4.	August 5.
A.....	0	0	0	0
B.....	99	100	78	81
C.....	74	69	54	49
D.....	0	0	0	0
E.....	43	34	21	17

In some cases a concentration of *Echinarachnius* blood that has *no* effect on the fertilization of *Echinarachnius* eggs by its own sperm will not give a single membrane or cleavage with heavy *Arbacia* sperm suspension. Again, shed eggs of *Echinarachnius* are superior to eggs cut out of the ovaries for cross fertilization. Also, after thorough washing, eggs cut out of the ovaries and fertilized with *Arbacia* sperm yield a higher per cent. of cleavage. I interpret these facts as indicating that it is the presence of blood that makes cross fertilization difficult. Blood thus acts in cross fertilization as it does in straight fertilization; the differences are quantitative only. For *Arbacia* sperm enter *Echinarachnius* eggs

in the presence of blood, but they set up no reaction. In my 1917 series of *Echinarachnius* eggs, heavily inseminated with *Arbacia* sperm, this is clearly shown in the sectioned material. Moreover, these eggs are viable; though literally studded with *Arbacia* sperm, they are capable up to twenty-four hours later on insemination with *Echinarachnius* sperm of giving development of a high degree of normality.

It should be noted that in these experiments clean shed sperm of *Arbacia* was used. If the sperm be obtained from the testes admixed with blood, the per cent. of cross fertilization is reduced. This has been repeatedly observed. In some cases, indeed, the shed sperm may give around 30 per cent. fertilization and the sperm from the testes no fertilization. I believe that this is due to the toxicity of *Arbacia* blood. This toxicity is well known from Lillie's observations. I have likewise mentioned elsewhere that *Arbacia* blood is markedly toxic for *Nereis* eggs.

Similarly, mixtures of shed *Arbacia* sperm and shed *Echinarachnius* sperm exhibit no antagonism; eggs of either form or of both dropped into such sperm mixtures fertilize. With mixtures of sperm cut from the testes the results are different, for such mixtures cut down the per cent. of cleavage. In one experiment made with mixtures of shed *Arbacia* sperm mixed with shed *Nereis* sperm there was no sperm antagonism, since eggs of each form developed upon inseminations from the mixture.

These, then, are the results of inseminating eggs of *Echinarachnius* in its body fluid or own blood.

We may conclude: (1) Blood blocks straight fertilization. (2) Blood blocks cross fertilization. (3) Blood blocks both straight and cross fertilization after the spermatozoa stick to the eggs or enter them and not by preventing the attachment of spermatozoa to the eggs.

These conclusions admit of certain suggestions concerning the nature of specificity in the fertilization reaction. We may discuss these briefly.

III.

The block to cross fertilization is cortical. As Lillie says: "The various methods used to induce hybrid fertilization—staling of

eggs, high concentration of sperm, use of alkalies or other chemicals—have therefore this one feature in common, that they destroy the chemical or physical integrity of the cortex of the egg" (Lillie, '19, page 219). Specificity in fertilization thus manifests itself in the cortex of the egg.

But specificity in fertilization is not absolute, but relative. This fact would seem to indicate that the results of straight and of cross fertilization are due to quantitative, not qualitative, differences in the cortical response to insemination; species sperm more readily than foreign sperm overcome the same resistance to fertilization set up by some cortical substance or condition. The question, therefore, comes down to this: What in the cortex is responsible for the block to fertilization, whether by species or foreign sperm?

In the first place, most methods used to induce cross fertilization in echinids hasten the loss of fertilizin. Thus staling is an easy method for the removal of fertilizin. Eggs allowed to stand or repeatedly washed lose their fertilizin content. Washing the eggs rapidly with dilute sea-water brings about a loss of fertilizin. Dense sperm suspensions rapidly bind available fertilizin. I venture the opinion that heat hastens the loss of fertilizin also.

If, now, we postulate that specificity in fertilization is wholly due to the presence of fertilizin, then must we also take the next step, namely, that cross fertilization is most successful when the fertilizin is reduced? That is, fertilizin is necessary for straight fertilization, but a block to cross fertilization; certain kinds of artificial parthenogenesis (heat, for example, on *Nereis* egg) depend upon the presence of the fertilizin in maximum concentration; certain eggs lose their capacity for fertilization by species sperm very rapidly (*Platynereis*); but with foreign sperm the case is otherwise—it can fertilize after an egg is no longer capable of response to artificial stimulus or that of species germ. But might not specificity in fertilization be accounted for in part on the basis of the data presented in this paper? This would mean at least with the knowledge at hand that specificity in fertilization is due in part to the blood, since the presence of blood blocks fertilization by species or foreign sperm.

When species sperm comes in contact with an egg, it gains entrance and fertilizes against the blood present. The greater the amount of blood, the more difficult the fertilization. Indeed, the blood may actually inhibit fertilization in every egg. Therefore, dense sperm suspensions must be employed for fertilization in the presence of blood rich in inhibitor. The blood inhibitor acts by binding the fertilizin so that the fertilizin can not react with the egg receptors. Heavy insemination insures fertilization perhaps by increasing the chances of some spermatozoa locating fertilizin free of blood inhibitor. Or in heavy insemination the onslaught of numerous sperm brings it about that the fertilizin shakes free the inhibitor.

The blood slowly leaves the egg as it lies in sea-water. But the fertilizin also goes. Hence while the egg is losing inhibitor it is also losing fertilizing power. The blood is perhaps never an irremovable block to species sperm; however, though present in but a trace, it serves to block foreign sperm. In staling, therefore, what results is not only loss of fertilizin, but also loss of blood. The loss of blood makes possible cross fertilization.

What is true of staling is doubtless true of other methods for obtaining cross fertilization—heat, use of alkali, and of dilute sea-water; they serve to remove the blood block. The fertilizin remains albeit in reduced quantity. Whenever an egg is capable of fertilization it possesses the fertilizable substance. And it is safe to assume that an egg that will not respond to its own sperm will not cross fertilize.

From this point of view, then, fertilizin is not the only factor in specificity. It is specific since it engages species sperm against the inhibition of blood. But the blood is an aid to specificity, since it blocks all sperm, species sperm least of all.

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THE FERTILIZATION-REACTION IN *ECHINARACHNIUS PARMA*. VIII.

Fertilization in Dilute Sea-water.

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The writer has shown,[2] by means of experiments made in 1920 at the Marine Biological Laboratory, Woods Hole, Mass., that a given dilution of sea-water with tap water which leaves the uninseminated egg of *Echinarachnius parma* intact is rapidly injurious to the inseminated egg during its process of membrane separation. After membrane separation the egg is likewise resistant to the action of the dilute sea-water. Certain experiments were also made during the same season to learn if it is possible to procure fertilization and development in diluted sea-water to which the inseminated egg during membrane separation is susceptible. These experiments were repeated during May and June of the 1921 season at the laboratory with essentially the same results.

In experiments made to discover if in a given dilution of sea-water, which is injurious to the inseminated egg of *Echinarachnius* during the period of membrane separation, it is possible to fertilize the egg, it was soon apparent that cleavage and normal gastrulation are not possible in a dilution which permits membrane separation. Thus during the period June 21 to June 28, 1921, inclusive, eggs from twenty-four females were inseminated in normal sea-water and in sea-water of varying dilutions. In all cases due precautions were taken as to the bulk of eggs, quantity of solution, and density of the sperm suspensions used in order that as far as possible conditions be made uniform. The results obtained with 95 per cent. sea-water (95 parts sea-water plus 5 parts tap water) and the dilutions ranging from this to 80 per cent. sea-water (80 parts sea-water plus 20 parts tap water) may be

[1] Zoölogical Laboratory, Howard University.

[2] Am. Jour. Phys., 1922, 61, 516.

summarized briefly as follows: In 95 per cent. sea-water eggs fertilize normally as revealed by the per cent. of membranes formed. They cleave and gastrulate in this dilution. In most cases the eggs are scarcely to be distinguished from eggs inseminated in normal sea-water. In 90 per cent. sea-water membranes are normal and cleavage fairly normally; but these eggs form about 10 per cent. exogastrulæ. In 85 per cent. sea-water the per cent. of membranes is close to normal—95 per cent. or slightly more. The cleavages show some abnormalities and the per cent. of exogastrulæ is greater than in 90 per cent. sea-water. In 80 per cent. sea-water the per cent. of cleavage falls and the per cent. of exogastrulæ increases.

The experiments indicated that the dilution made up with 75 parts sea-water plus 25 parts tap water is the lowest which permits cleavage. In this the gastrulæ are very abnormal. We may, therefore, turn our attention to the experiments made with this and greater dilutions of sea-water. Eggs inseminated in dilutions greater than those that permit cleavage separate membranes. Even in the dilution made up of equal parts tap water and sea-water some membranes separate, though these are often hard to see. Eggs, therefore, will respond to insemination with at least abortive cortical changes, though they can not cleave. I cite now one experiment which was made—as the majority of these experiments—on the eggs of three females.

June 21, 3:30 P.M. Six series of dilute sea-water, each consisting of 6 members, as follows:

No.	1.	2.	3.	4.	5.	6.
Parts sea water	50	55	60	65	70	75
Parts tap-water	50	45	40	35	30	25

Equal portions of uninseminated eggs from each of 3 females (*A*, *B*, and *C*) put in each dilution of sea-water to make Series I*A*, I*B*, and I*C*, II*A*, II*B*, and II*C*.

Series I*A*, I*B*, and I*C* uninseminated.

Series II*A*, II*B*, and II*C* inseminated with same quantity of sperm from one male. Inseminations in each series 5–25 seconds after eggs in dilute sea-water.

Controls: uninseminated eggs in normal sea-water; inseminated eggs in normal sea-water.

The eggs were examined at intervals. The next day, 7:50 A.M., the uninseminated eggs (Series I*A*, I*B*, and I*C*) showed an average of less than 10 per cent. cytolysis. The history of Series II. is given in the table below (Table I.):

TABLE I.

PER CENT. OF MEMBRANES AND OF CLEAVAGE IN EGGS OF *Echinarachnius* IN-IN SEA-WATER OF VARYING DILUTION.

No.	Amount of Sea-water in 100 Parts of Sea-water plus Tap Water.	Per Cent. of Membranes in Eggs from the 3 Females.			Per Cent. of Cytolysis Two Hours after Insemination.			Per Cent. of Cleavage 5 Hours after Insemination.		
		A.	*B.*	*C.*	*A.*	*B.*	*C.*	*A.*	*B.*	*C.*
1	50	0	2	1	18	4	9	0	0	0
2	55	0	2	0	4	2	1	0	0	0
3	60	7	7	7	0	3	0	0	0	0
4	65	5	22	20	0	1	0	0	0	0
5	70	14	63	47	0	3	0	0	0	0
6	75	60	95	87	0	2	0	5	16	4
Uninseminated control	100	0	0	0	0	0.1	0	0	0	0
Inseminated control	100	100	98	100	0	0.5	0	100	97	100

This experiment indicates that eggs inseminated in dilutions of sea-water may separate membranes, though they do not cleave. It would, therefore, be erroneous to assert—if membrane separation be the criterion for fertilization—that eggs that do not cleave have not been fertilized. Rather the failure to cleave is due to the action of the dilute sea-water in interfering with the cleavage mechanism—particularly with the activity of the hyaline plasma layer. There is no question here of "partial fertilization"; it is wholly a question of incomplete cleavage. This is important for the experiments that we may now consider.

In the experiments now to be considered it was repeatedly found that eggs may be inseminated in a dilution that is destructive to the egg which is inseminated in sea-water and exposed to this dilution during membrane separation. The criterion of this destructive action is the differential cytolysis of uninseminated and inseminated eggs before, during, and after membrane separation.

With cleavage, however, the case is quite different. That is, the per cent. of cleavage of eggs inseminated in a given dilution is no higher than that of eggs inseminated in sea-water and transferred to the dilution before, during, or after membrane separation. There might be a slight indication that eggs exposed during membrane separation show a lower per cent. of cleavage. I believe, however, that my figures on this point are not decisive.

I give now one experiment to show the per cent. of cytolysis in eggs inseminated in dilutions of sea-water as compared with that of eggs inseminated in sea-water and placed in the dilutions before, during, and after membrane separation.

June 27, 11:25 A.M. Following dilutions prepared:

No.	1.	2.	3.	4.	5.	6.
Parts tap-water	50	45	40	35	30	25
Parts sea-water	50	55	60	65	70	75

Five dishes for each of the six dilutions containing 10 c.c. each. Thus 5 series of 6 numbers each.

Drops of uninseminated eggs from one female added to dilutions as follows:

Series 1: 1 drop of eggs in each dilution.

Series 2: 1 drop of eggs in each dilution; inseminated immediately.

Series 3: Drop of eggs to each dilution 15 seconds after insemination in sea-water.

Series 4: Drop of eggs to each dilution during the period of membrane separation 35 seconds after insemination in sea-water.

Series 5: Drop of eggs to each dilution two minutes after insemination in sea-water.

Controls: Uninseminated eggs in normal sea-water; inseminated eggs in normal sea-water.

The results of this experiment follow:

12 M.

Series 1: Less than 1 per cent. cytolysis.

Series 2: As in Series 1.

Series 3:
(12:00 M.)

No.	Dilution (Parts of Sea-water in 100 Parts of Solution.	Per Cent. of Eggs Intact.	Per Cent. of Eggs Cytolyzed.
1	50	79	21
2	55	77	23
3	60	89	11
4	65	98	2
5	70	97	3
6	75	100	0

Series 4:
(1:00 P.M.)

1	50	50	50
2	55	47	43
3	60	85	15
4	65	80	20
5	70	90	10
6	75	97	3

Series 5:
(1:30 P.M.)

1	50	69	31
2	55	83	17
3	60	86	14
4	65	97	3
5	70	99	1
6	75	98	2

Controls: Uninseminated in sea-water—1 per cent. cytolysis.
Inseminated in sea-water 99 + cleavage.

It is clear from this experiment that eggs exposed to a dilution of sea-water during membrane separation cytolyze in slightly higher per cent. than eggs inseminated in the dilution. Similarly, eggs inseminated in normal sea-water and exposed before or after membrane separation cytolyze in slightly lower per cent. than eggs exposed during membrane separation. The cleavage per cent. of *surviving* eggs is about the same.

If we hold that membrane separation is a criterion of fertilization, then eggs inseminated in a given dilution of sea-water are fertilized whether they cleave or not. This admittedly is not a strong case, but it gives some support to the position that the fertilization reaction is practically instantaneous. It is not fertilization that is checked by the action of dilute sea-water, but the reactions which set up by fertilization lead to cell division.

REACTIONS OF CELL-BODIES AND PSEUDOPODIAL FRAGMENTS OF *DIFFLUGIA*.[1]

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INTRODUCTION.

The purpose of this paper is to record a number of observations which were prompted by an incident occurring on October 7, 1919, while one of us (Kepner) was watching a *Difflugia pyriformis* Ehrenberg under the compound microscope. The animal suddenly retracted an unusually long pseudopod, and in doing so its distal end was torn off. In a short time the fragment began to show amœboid movement; meanwhile the animal sent out other pseudopods, and within a few minutes the two masses of protoplasm made contact and fusion took place. This naturally called to mind the work which had been done by others on regeneration and restitution; therefore a series of experiments were undertaken in order to find out to what extent, if any, this phenomenon was exhibited in *Difflugia*.

It is generally known that many animals possess conspicuous powers of regeneration. For example: snails are able to regenerate tentacles; earthworms, flatworms, and polyps may have their bodies severed and each fragment be able to restore the parts that it lacks to complete a body. Verworn (1892) observed that severed fragments of *Orbitolites* would occasionally adhere to the ends of parental pseudopods. Jensen (1896) pointed out that *Orbitolites* and *Amphistegina* (belonging to the order *Foramifera*) would sometimes recover fragments belonging to themselves and make them again a part of their bodies. Wilson (1907, '11) has shown that the tissues of sponges broken up or macerated by passage through the meshes of bolting cloth, so that the component cells

[1] This is the first of a series of contributions on this subject. More extensive observations and experiments are now being conducted by the junior author with other genera of Rhizopods, as well as with the genus *Difflugia*.

are separated and distributed in water, will reassemble and form new individuals.

The conditions of life under which the above animals live impose a risk of the body parts. *Difflugia* is protected, to a certain extent, from external environments by its shell, and perhaps also by the formation of a plug of mucus about the mouth of its shell when unduly stimulated. Nevertheless, in sending out long, slender pseudopods, it, too, runs a risk of losing some of its protoplasm, for, as recorded by Verworn (1890), when the projecting pseudopods are strongly stimulated, they are frequently jerked back so suddenly as to whip off their ends.

The Organisms.

Various species of *Difflugia* were used in these experiments, all of which gave similar results. However, it was found that *D. acuminata, D. corona, D. pyriformis, D. spiralis,* and *D. vulgaris* were more favorable for study because of the nature of their pseudopodial formation, and also because they commonly drag themselves after their advancing pseudopods with the shell in a reclining position, so that the mouth of the shell and the bases of the pseudopods can be seen easily. This feature also affords a better opportunity for cutting off pseudopods without danger of striking the shell.

Methods.

(*a*) *Collecting.*

The animals were collected in small puddles in a cow pasture near the University of Virginia. These puddles were near a fresh stream and frequently obtain a supply of fresh water, due to rains, and the overflowing of the banks of the stream. In the same puddles other protozoa, fresh-water crustacea, and various algæ are also found. The brown ooze and vegetable matter on the bottom of these little pools were carried to the laboratory in fruit jars, then allowed to stand for several hours, after which the surface of the sediment, along with sufficient water to fill the vessel to a depth of approximately five millimeters, was transferred to Petri dishes. The organisms can be kept in this way for several weeks, provided fresh water is added occasionally. In these experiments no attempt was made to maintain pure line cultures.

(*b*) *Apparatus and Technique.*

Individuals can easily be isolated for study by placing a Petri dish, containing the organisms, under a low-powered binocular, locating the animal desired, and transferring it by means of a narrow-mouthed pipette to a plain slide containing a large drop of spring (or distilled) water. The amputations were made with fine glass needles, which can readily be drawn in a small hot flame. It is essential to use hard elastic glass for this purpose.

At first the operations were made under a binocular, and the slide containing the organism was then transferred immediately to a compound microscope for observation, but later operations were made under the compound microscope, using a 32-mm. objective and a No. 10 eyepiece (B. & L.). The latter method is altogether more satisfactory, since it enables the observer to keep sight of the performance from the very beginning, and also avoids any jarring or shifting of the protoplasms, which might take place while transferring the slide. Practically all of the observations were made under the 16-mm. objective and No. 10 eyepiece. Most of the distances given in this paper were estimated either in terms of the diameter of the field or in terms of the diameters of the shells of the individuals concerned. A later and more satisfactory method is to use a camera lucida and trace the outlines of the experimental objects at definite intervals. By this method exact record can be kept of the relative sizes and positions, as well as the paths followed by each body.

I. Is Reunion of Cell-body and Fragment the Usual Occurrence?

In order to determine this we performed approximately one hundred experiments, the majority of which gave positive results. The few negative cases observed were apparently due to one of the following causes: (*a*) Severe injury to cell with consequent failure on its part to extrude pseudopods, (*b*) separation from fragment by too great a distance, (*c*) evaporation of water before completion of reaction. These results have been repeatedly confirmed by graduate students in this laboratory. Since mucus is involved in the organisms' locomotion, it occurred to us that the

separated parts might follow a mucous trail, or even invisible strands of protoplasm, and thus contact would be effected. For this reason, in most cases after a pseudopod was severed the cell-body was moved to a considerable distance, and yet the fragment was recovered. Again the cell-body has been moved in a wide detour around on another side of its fragment, after which it would approach without regard to the course along which it had been pushed. The mouth of the shell has been placed in every angle to the fragment from zero to one hundred and eighty degrees; nevertheless the fragment is usually recovered. To give the details of each experiment would require too much space and necessitate repetition of similar results; therefore, the following two observations have been selected as typical of the usual reaction:[1]

On July 24, 1920, the end of a pseudopod was cut from a *Difflugia vulgaris* by Mr. E. Paylor, of this laboratory, after which the cell-body was immediately moved 500 micra away from it, the mouth of the shell being directed away from the fragment, as shown in Fig. 1—*A*. Within a minute the animal, which had withdrawn into its shell after the operation, began to send out two pseudopods. After these pseudopods had been extended to a distance of about fifty micra, their ends adhered to the glass slide on which the animal was lying. Then by a movement somewhat similar to a hand-spring the body proper was thrown over the extended pseudopods, so that now the mouth of the shell was pointed toward the fragment (Fig. 1—*B*). The animal then began to move in the direction of the fragment, moving in a counter-clockwise curve as indicated by Fig. 1—*C*, *D*, *E*, and *F*. This movement was continuous until the fragment was encountered and reappropriated through fusion of the two masses of protoplasm (Fig. 1—*G*).

The second example given to illustrate the phenomenon of fusion is perhaps more interesting, for in this case more than one fragment was involved. In this experiment exact record of the time

[1] We realize that the conclusions drawn in this paper would be more convincing if we published our data in extenso, but since this is a preliminary report of observations on a subject which is now being actively investigated with a view to further and more extensive publications, it seems best to present our results here by means of typical experiments and general statements regarding the entire investigation.

at which each event took place was made. At 11:38 A.M., June 10, 1920, a small fragment, *c*, Fig. 2, was cut off from a *Difflugia spiralis*. The animal immediately withdrew its extended pseudopods, but not completely within its shell. It then sent out two new pseudopods, and at 11:39 A.M. the ends of both these were cut off, *a* and *b*. The animal was so stimulated by this second operation that it quickly retracted its pseudopods, and in doing so was turned through an angle of ninety degrees, so that now its mouth lay directed at right angles to the fragments, which were lined up in a row. In a short time the animal extended a short pseudopod which curved around alongside the neck of its shell and fused with *a* at 11:40½ A.M. The cell-body was then moved 600 micra away and placed so that the side of its shell was toward the remaining two fragments which had been left in their original positions. By 11:45 A.M. it had advanced again and fused with fragment *c*. The animal was then stimulated with a glass rod, so that it withdrew into its shell again; but by 11:45¾ A.M. it had sent out another pseudopod which fused with fragment *b*. Thus within seven and three quarter minutes from the time the first fragment was cut off the animal had picked up and reappropriated all three fragments, though during the interval it had been stimulated by the observer so violently as to make it contract four times, and at one time it was moved six times its shell's diameter away, through which distance it had to travel in order to get back to its missing parts.

It has not been determined exactly what the maximum range of positive response is—*i.e.*, the limit within which fusion always occurs. One positive case was observed in which the cell-body and fragment were separated by one and one half millimeters. But chance wandering might have brought the animal within the positive range. Be this as it may, chance can hardly explain the large percentage of positive observations made. When the two elements were separated by great distances, we encountered a small percentage of negative reactions. However, in every case which we observed where the cell-body and fragment were separated by a distance of 500 micra or less fusion always took place, unless the cell-body was injured. It seems, then, that reunion is

the usual thing which takes place, if the missing parts be not too far removed.

II. Is the Phenomenon a Process of Feeding?

From the experiments described in the preceding section the conclusion was drawn that *if a fragment of protoplasm be severed from a Difflugia, it is usually reappropriated.* The question naturally arises: Does the animal react to its fragment as a piece of food, or do the two coalesce? If the fragment is taken up as food, we would expect to see: (*a*) formation of a food vacuole and enlargement at site of ingestion, (*b*) a similar reaction to fragments from closely related forms, and (*c*) no sharp distinction as to whether or not the fragment showed visible signs of life, such as movement.

(*a*) In some of these observations the animals were in such a position at the time contact was made that it was impossible to see what took place on account of the shell, but in the majority of cases the view was unobstructed, so the entire process could be witnessed. The usual reaction is approximately as follows: As the advancing pseudopods of the cell-body near the fragment they usually cease to become attached to the substratum, as in the ordinary process of locomotion; thus movement on the part of the animal is slowed down. If at this time only one pseudopod is extended, smaller secondary ones may be sent out near its base or from the mouth of the shell. The primary pseudopod may make direct contact with the fragment, or it may form a small cup into which the fragment is fitted, or a niche may be formed by the extrusion of one or more secondary pseudopods, and contact is made in the niche thus formed. If there are two or more primary pseudopods, they may encircle the fragment collectively, or only one of them may take part in the reaction. When contact is made there is ordinarily a disturbance of the protoplasmic surfaces, which varies from a mild local shock to a violent contortion involving the entire (visible) protoplasm. When the "contact shock" is very great it is impossible to observe what becomes of the fragment, but when it is slight the granular ectoplasm constituting the fragment can be seen streaming into the pseudopod and

becoming disseminated, as though the fragment were a lateral branch which the pseudopod was withdrawing. There is no sign of incoherency or enlargement at this place (see Fig. 5—*A, d*).

(*b*) The reaction toward foreign fragments is different, as will be brought out later.

(*c*) On many occasions it was observed that after fragments had been separated from their cell-bodies for a long time they would cease to move, become rounded off, and take on an opaque granular appearance. The time required to bring about this change depends on several conditions, particularly on the size of the fragment; a large fragment retaining its power of movement longer, other things being equal. Though it has not been determined experimentally, it is reasonable to presume that the physiological condition of such a fragment is changed as well as its appearance; yet its molecular composition can hardly be so altered as to change its food value. Though over twenty-five observations have been made on the reaction between *Difflugia* and its fragment, which had assumed a granular, opaque, spherical condition, not a single instance was found in which the animal picked up such a fragment. The following example is illustrative of the reactions obtained:

On June 10, 1920, at 11:47 A.M., a large pseudopod was cut from a *Difflugia spiralis* and the cell-body was then moved out of the field. The fragment moved about for fifteen minutes, then gradually ceased and assumed a spherical shape. By 12:15 half of the bulk constituting this mass of protoplasm showed disintegration to such an extent that it had changed from the spherical condition and was scattered, in an uneven manner, around the portion which still retained its rotundity. At this time the cell-body was brought up and the mouth of its shell placed in contact with the fragment. The animal soon extended two pseudopods which came in contact with the fragment in the manner indicated in Fig. 3—*A*. For two minutes the animal remained practically passive, then it began to move forward, taking with it the portion of the fragment which was still in a spherical condition. This was held by a secondary pseudopod which had formed an imperfect cup around it (Fig. 3—*B*), the disintegrated portion being left behind. Movement was continued in the same direction until

the animal had traveled twice the distance of the diameter of its shell. But at 12:22 P.M. it rejected the portion of the fragment which it held and reversed its course, leaving the fragment behind.

From our observations, then, it seems evident that severed fragments are not taken up by the cell-body as food, but that the separated bits of protoplasm recombine with the original mass and take up their function as though nothing had happened, provided disintegration processes have not set in before contact is made.

III. Does Fusion Take Place at Any Point Along the Pseudopod?

A *Difflugia* progresses by extending pseudopods (sometimes they are very long and slender), the ends of which become attached to the substratum. With this in mind, one would naturally expect that the end of an advancing pseudopod would first make contact with a fragment which the animal was approaching. On the contrary, this has never been observed. In the last section attention was called to the fact that when nearing a fragment the ends cease to become attached. At this stage they usually wave around in the medium, and further progression is made either by this waving motion, or else by smaller pseudopods near the mouth of the shell. Though not all cases were quite so clear cut, the following example illustrates the principal region of a pseudopod involved:

On October 7, 1919, a *Difflugia pyriformis* was observed extending a large pseudopod toward its fragment *a* (Fig. 4—*A*). In the meantime the fragment was moving in the direction shown by *e*, *f*, and *g* (Fig. 4). After its end had passed well beyond the fragment, the pseudopod was bent toward the fragment until its side had come in contact with the rear end of the moving ectoplasmic body. When this contact was made, the animal threw out an intimately fitting cup around the posterior lobe of the fragment (Fig. 4—*B*, *g*, and *g'*). Immediately the protoplasm, which had been detached, flowed back into the pseudopod, without causing any perceptible increase in its diameter.

In all cases which we observed fusion took place along an extended mid-region, neither at the tips nor at the bases of pseudopods.

IV. Does the Fragment Take an Active Part in Restitution?

If fusion between a cell-body and its fragment is true restitution as has been claimed, one should not be surprised to see the fragment taking an active part in the process. With this in view, especial attention was given to the movements of fragments. Without some object to mark the original positions, it is very easy to confuse distances traveled by each body when two are approaching each other. Therefore, when observations were made without the use of a camera lucida to plot the positions, care was taken to avoid falling into this error. Whenever fusion took place, the protoplasm constituting the fragment seemed to flow into, and commingle with, the protoplasm of the cell-body. But it was not always possible to observe an appreciable movement on the part of a fragment toward its cell-body before contact was made. However, on many occasions this could be seen. The two following accounts are typical of the more striking reactions of this sort:

On November 28, 1919, a fragment was cut from a *Difflugia spiralis*, after which the cell-body was moved 1,500 micra distant. Immediately after separation the fragment had the shape of an elongated ovoid, then its contour changed continually for the next thirteen minutes (Fig. 5—3, 4, 5, 6, 7, 8, 9, etc.). Each figure represents the number of minutes that had elapsed since the operation took place. During the first twelve minutes its movement seemed to be indefinite, as indicated in the diagrams, but by this time the cell-body had advanced until the end of its nearest pseudopod was within 150 micra of the fragment. From this time there was a decided movement on the part of the fragment in the direction of the cell-body. As soon as the fragment had come within reach of the long pseudopod, which the cell-body was extruding, movement by the latter slowed down, and the distal end of the pseudopod was lifted. Under this the fragment passed. The fragment continued the approach, and two minutes from the time it had come within the radius of the animal's pseudopod it had traveled about twenty micra and reunited with its cell-body (Fig. 5—*A*, *a*, *b*, *c*, and *d*).

On November 8, 1919, a large fragment was cut from a pseudo-

pod at 10:44 A.M. This soon became greatly contorted, and from its tangled mass two amœboid fragments emerged. The water became shallow soon after the operation and it was necessary to add more, which separated the fragments a considerable distance from the cell-body. At 11:16 A.M. the fragments were placed in the vicinity of the cell-body again (Fig. 6—*A*, *a*, *b*). At 11:23 A.M. fragment *a* had shifted its axis so that it now lay directed toward the mouth of the animal's shell. Fragment *b*, during the same period, traveled to position *x*. The cell-body was now pushed up to the relative position shown at *B* without disturbing the fragment. The anterior tip of fragment *b* was immediately shifted so that it was now directed toward the new position of the shell's mouth. The entire fragment had advanced to position *y* by 11:27 A.M. At this time fresh water was added to the slide, and in doing so the fragments were lost.

That such enucleated fragments show a positive response to stimulus, whatever it be, seems unquestionable. The importance of this is, perhaps, emphasized by the fact that all fragments involved in our observations were not simply enucleated bodies of protoplasm, for endoplasm did not constitute a portion of any of them. They were, therefore, *enucleated ectoplasmic fragments.*

V. Will Two Enucleated Fragments Fuse with Each Other?

Having observed the readiness with which fusion took place between *Difflugia* and its ectoplasmic fragment, the question suggested itself—*Is there a tendency between two pieces of protoplasm to fuse when no restitution of a cell would result from such a fusion?* Not a sufficient number of experiments has been performed to warrant an emphatic statement in regard to this point. But at least twenty observations have been made in which two or more fragments from the same organism were involved. In these experiments the fragments were only ectoplasmic in composition and varied in size from five to twenty micra in diameter. The results of these experiments can be illustrated by the following example:

A large and a small fragment were cut from a *Difflugia spiralis*, after which the animal was removed from the slide. The frag-

ments were separated by the distance of about fifty micra, and for two minutes they moved around quite actively, but no progress was made toward uniting. They were then placed in contact, by using a glass needle, so that now they were in the positions shown in Fig. 7—*m*. This contact was a very close one, the adjoining surfaces being very extensive and the line of demarcation quite indistinct, yet discernible. Ten minutes later the activity of the fragments had begun to cease and they showed signs of rounding up into spherical bodies, each piece into its respective sphere (Fig. 7—*n*). After ten more minutes the spherical condition had been attained and the pieces showed only a small point of contact (Fig. 7—*o*).

Thus, after having been placed in direct contact, there was a failure on the part of these two fragments of ectoplasm to unite—though they had come from the same cell. It seems, then, from our observations, that enucleated ectoplasms do not fuse with each other.

VI. Will *Difflugia* Fuse with Pseudopodial Fragments Belonging to an Individual of a Different Species?

(*a*) *As Food.* (*b*) *By Protoplasmic Union.*

The criteria for determining whether or not the fragments serve as food have been set forth in a previous part of this paper. However, since the reaction of an individual toward fragments belonging to closely related forms was proposed as one method for determining this, these experiments were designed to shed some light on this subject. Furthermore, it occurred to us that in such organisms an individual might fuse with foreign fragments in the same manner described for cell-bodies and their own fragments. The results obtained were such that it will not be necessary to make a distinction between (*a*) and (*b*) in discussing the reactions. The method which we employed was to take two *Difflugia* of different species and place them near each other in a drop of water, and as soon as one of the animals had extruded a long pseudopod it was cut off and the animal then removed from the drop, leaving its fragment and the other individual behind. Attempts were made in this way to cross the protoplasm of all the various species of *Difflugia* previously mentioned, viz.: *D. acumi-*

nata, D. corona, D. pyriformis, D. spiralis, and *D. vulgaris.* While only two or three observations were made on each combination, the results were all negative. Therefore, we feel a degree of assurance in asserting that cross-fusions do not normally take place. The following is one of the most striking observations made on this type of reaction:

On June 10, 1920, a fragment was cut from a *D. spiralis* (animal *A*). This animal was then moved from the field, and a *D. vulgaris* (animal *B*) was brought into contact with the fragment. This contact remained for three minutes, after which animal *B* left the fragment and moved from it to a distance of 200 micra; but within two minutes it had turned around, retraced its course, and made contact with the fragment again. This time such close contact was made that it appeared as though fusion had resulted. Such intimate relations continued for five minutes, then another separation, animal *B* leaving the fragment for the second time. After it had traveled about 300 micra it again turned and came back to the fragment of foreign ectoplasm, making a third contact in about three minutes. This time the two objects did not adhere so closely as on previous occasions. Three minutes later animal *B* moved away from the fragment again, this time remaining away four minutes before returning to make the fourth contact. On this occasion the animal made only slight contact with the fragment, moving off again after a few seconds. When animal *B* had traveled approximately 100 micra, it reversed its course and seemed about to approach the fragment another time. However, before reaching it the animal reversed its course and did not return any more. During the whole procedure (an interval of twenty-six minutes) there was no positive reaction on the part of the fragment of *D. spiralis* toward the *D. vulgaris.*

While it is possible that by using different media different reactions may be obtained, it seems that under conditions comparable to their normal habitat one species of *Difflugia* will not assimilate protoplasm belonging to another species.

VII. Will Individuals Fuse with Protoplasm from Other Individuals of the Same Species?

Being unable to get cross-fusions between different species, we

next sought to determine whether or not one individual would fuse with a pseudopodial fragment from another individual of the same species. In order to make conditions similar to those under which organisms have been repeatedly observed to pick up their own fragments, a portion of the protoplasm was removed from the animal concerned before placing a fragment from another individual near it. The following reaction is an example of what commonly happens when dealing with wild cultures:

Two specimens of *D. spiralis* (*A* and *B*) were placed in the same drop of water, and a fragment was cut from animal *A* at 10:24 A.M., July 14, 1921. This fragment was carried out of the water as it clung to the glass filament with which the cutting was done. Animal *B* was now dragged up to the position shown in drawing (Fig. 8). At 10:29 A.M. fragment *c* was cut from animal *B* without disturbing the position of either cell; the operation did not even cause animal *A* to withdraw its large pseudopod. Specimen *B* was immediately carried from the drop. Animal *A* turned away from the fragment to position *A'* and moved off, but a little later it was moved back to less than half its shell's length from the fragment, only to move away again. For the third time animal *A* was placed near the fragment of foreign ectoplasm, and though one of animal *A*'s pseudopods passed directly over it, there was no reaction either on the part of animal *A* or the fragment from animal *B*.

Other reactions have been observed in which individuals did fuse with fragments from animals of the same species. However, in all reactions of this sort the organisms were obtained from the same culture. There are two possible explanations for this: (*a*) *The individuals were closely related by having a recent common ancestor.* (*b*) *By living in the same surroundings, the environmental influences have acted upon both organisms in such a way as to cause an identical physiological state.* This is left as an open question, upon which further work is being done.

Discussion.

Verworn (1892) cut off pseudopods from *Orbitolites* and observed that after the severed masses had rounded up into little droplets they would adhere to the *ends* of pseudopods extended by

their cell-bodies. Soon thereafter the masses of severed protoplasm would bend at the points of contact and the contents would flow up the pseudopods. Jensen (1896) pointed out that in *Orbitolites* and *Amphistegina* chance crossing of pseudopods of different specimens may cause instantaneous shattering of the protoplasm concerned, and that the organisms would occasionally pick up the fragments belonging to themselves and make them again a part of their bodies. He was also able to get the same results by cutting off fragments of pseudopods. Furthermore, he observed that one species would react negatively toward fragments from other species. Later, in 1901, he made some further experiments on *Orbitolites* in which he obtained practically the same results. In the last paper he indicates that there is some response on the part of the fragments toward restitution. He calls attention to the fact that tendency toward restitution is stronger just before the severed fragments lose their power of movement. This does not correspond to our findings, and since he does not describe his experiments in detail, it is difficult to know whether or not they have much in common with those made by us.

There are many unicellular animals that may be cut into two parts and each part display vital phenomena. In the uninucleated forms the part, or parts, lacking a nucleus probably carry on only katabolism or destructive processes. In multinucleated protozoa we often find each mutilated fragment becoming a new individual—as, for example: when a *Stentor*, *Actinosphærium*, or *Arcella* is fragmented. Wilson (1907, '11) separated the cells of a sponge's body and found that these cells would become organized again into a new sponge. But this was restitution through segregation and reorganization of parts of a multicellular organism. In *Difflugia* we have the parts of a cell fusing to retain its former completeness. In this relation it is impressive to observe how definitely ectoplasmic fragments respond, in some cases, to their cell-bodies. As the cell shifts its position the fragment changes its course correspondingly.

Chemotaxis of some sort is probably involved in the attraction shown between cell-bodies and their fragments, but that point has not been determined. Neither mucous trails nor invisible strands of protoplasm are necessary for the protoplasmic reunion to take

place, for care has been taken to place the animals in such positions that this could be determined. Some substance may emanate from each *Difflugia,* as products of its individual metabolism, which furnishes the required stimulus.

An interesting feature of the reaction between an ectoplasmic fragment and its cell-body is presented by the fact that fusion has never been observed to take place at the tip of an advancing pseudopod, nor at its base, but always along an extended mid-region. This suggests that the ends of such pseudopods must be different from their sides. This, if well founded, carries the analysis of pseudopodial formation a bit beyond that presented by Hyman (1917), who showed that the younger pseudopods of an amœba have a different metabolic gradient from the older ones.

The ability of enucleated fragments to react to stimuli has been observed by others. Hofer (1889) showed that fragments of amœbæ lived for fourteen days; their movements were carried on, though somewhat modified. Verworn (1889) found that enucleated fragments of *Amœba, Difflugia, Lachrymaria, Polystomella,* and *Thalassicolla* live a long time and perform normal movements and normal reactions to stimuli. Minchin (p. 210) makes this statement: "Non-nucleated fragments may continue to live for a certain time; in the case of amœba such fragments may emit pseudopodia, the contractile vacuole continues to pulsate, and acts of ingestion or digestion of food that have begun may continue; but the power of initiating the capture and digestion of food ceases, consequently, all growth is at end, and sooner or later all non-nucleate bodies die off." Lynch (1919) observed that enucleated fragments of an amœba may move, respire, digest, respond to stimuli, and exhibit any activity which is dependent solely upon katabolic or destructive processes of protoplasm. The group of phenomena which they never show constitutes such processes as growth, regeneration, and division. Willis (1916), on the other hand, records that parts without a nucleus do not react as well as nucleated portions—for example: they do not orient with reference to a beam of light. Finally, Mast and Root (1916) say that "the fact that enucleated parts of amœba do not respond at all or respond in a haphazard fashion indicates, as Hofer ('90) concludes, that the nucleus acts as a regulatory center." Hofer,

Willis, and Mast and Root have found, therefore, that enucleated fragments of protozoa do not respond normally, while Verworn and Lynch find that katabolic activity is normal on the part of these enucleated fragments of protoplasm. In *Difflugia* we have found a response that is remarkably well regulated and very delicate. This is of especial interest because the fragments which we were dealing with were only ectoplasmic in composition.

We wish to avoid the implication that the regulated movements of these ectoplasmic fragments were effected without nuclear control, for the absence of nuclei in them does not preclude nuclear influence. Regulatory substances of nuclear origin might have been present in the ectoplasmic fragments at the time they were severed from the cell-body. For, as Minchin (p. 65) states, "in many protozoa, especially amongst the Sarcodina, as, for example, *Arcella, Difflugia,* and many other genera, the cell-body contains, in addition to one or more nuclei, extranuclear granules of chromatin, termed *chromidia,* which may be scattered in the cytoplasm throughout the cell, or may be aggregated in certain regions of the body to form 'chromidial masses' or 'chromidial nets.'"

Regulatory movements between a fragment and a cell were not maintained when a fragment was taken from an individual of one species and placed by the side of an individual of another species. This is in accord with the observations of Wilson (1907), who found that in dealing with dissociated sponge cells, "in contact two masses of the same specific protoplasm tend to fuse," while "unlike specific substances (protoplasms of quite different species) do not tend to fuse." In *Difflugia* protoplasms from two different species do not fuse or coalesce. Also the following quotation from Lillie (1920) can be applied to our findings on *Difflugia:* "If we put two different species of yeast or bacteria into the same culture-medium, each builds up protoplasm of its own kind; *i.e.,* each effects a special predetermined kind of chemical transformation in the materials which it incorporates from the surroundings. Each has the same external materials as its source of supply, but each transforms them in its own specific way, and hence builds up a special kind of protoplasmic structure which, having a special physio-chemical constitution or organization, exhibits corresponding special activities. The term specificity denotes this peculiarity."

This *peculiarity* has been carried a step further in *Difflugia,* for we find that in distantly related individuals of the same species the protoplasm has become so modified in an individualistic way that fusion will not take place between them. Loeb (1920) has recorded observations on the power of tissues to exhibit different reactions to different degrees of family relationship. Our experiments suggest a similar ability on the part of *Difflugia.*

Summary.

1. In *Difflugia* separated pseudopodial fragments are recovered by their cell-bodies, as evidenced by over one hundred experiments. The species included in these observations were: *D. acuminata, D. corona, D. pyriformis, D. spiralis,* and *D. vulgaris.* The distances by which the fragments and their cell-bodies were separated ranged from a few micra to 1,500 micra. The unfavorable conditions under which reunion does not occur are: (*a*) Injury to cell with consequent failure on its part to extrude pseudopods; (*b*) separation from fragment by too great a distance; (*c*) evaporation of water before completion of reactions.

2. The severed fragments are not recovered as food, but enter again immediately into the protoplasmic structure of the cell-body. Such fragments are not reappropriated after they show visible signs of death.

3. In our observations fusion never occurred at the ends of pseudopods, but always took place along an extended mid-region. This indicates a physio-chemical difference between the ends and the middle of such pseudopods.

4. In some cases the severed fragments, which were only ectoplasmic in composition, were observed to take an active part in restitution. Not only did they move toward, but apparently shifted, their line of approach to correspond with the changing positions of their cell-bodies.

5. Fusion has not been observed to take place between two enucleated fragments, even though they be placed in contact.

6. Fusion between an individual of one species and a fragment from an individual of a different species has not been observed. The phenomenon seems to be specific.

7. Observations have been made in which individuals of the same species, obtained from the same wild culture, showed a decidedly negative response toward each other's fragments. Yet in other instances such cross-fusions did occur. This latter suggests the possibility of close genetic origin.

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EXPLANATION OF PLATES.

PLATE I.

FIG. 1. *Difflugia pyriformis.* While at position *A* a fragment was cut off and left with spherical contour at position w. As cell-body moved to positions *B*, *C*, *D*, *E*, *F*, and *G*, fragment *w* changed shape and travelled as an ameboid body along path indicated by *x*, *y*, and *z*. *z* fused with the side of the upper pseudopod of cell-body at *G*.

FIG. 2. *Diffluga spiralis.* Fragment *c* cut off at 11:38 A.M. Fragments *a* and *b* cut from two pseudopods synchronously at 11:30 A.M. These fragments *a*, *b*, *c* now lay with reference to each other and to the cell-body as indicated. In a little time a slender pseudopod of cell-body came down by side of shell and *a* was appropriated. The cell-body was now dragged six diameters of its shell to left from *b* and *c*. By 11:45 A.M. it had taken up *c*. It was then disturbed with a glass rod; but at 11:45¾ A.M. it had emerged and taken up fragment *b*.

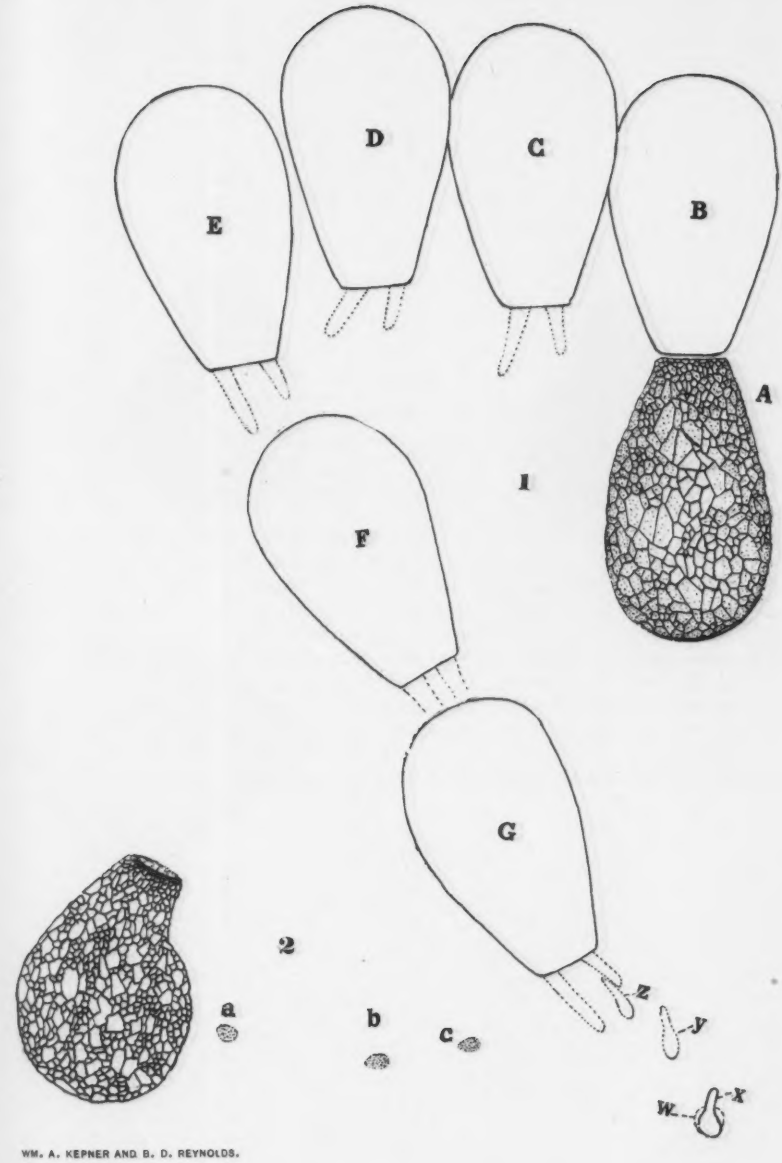

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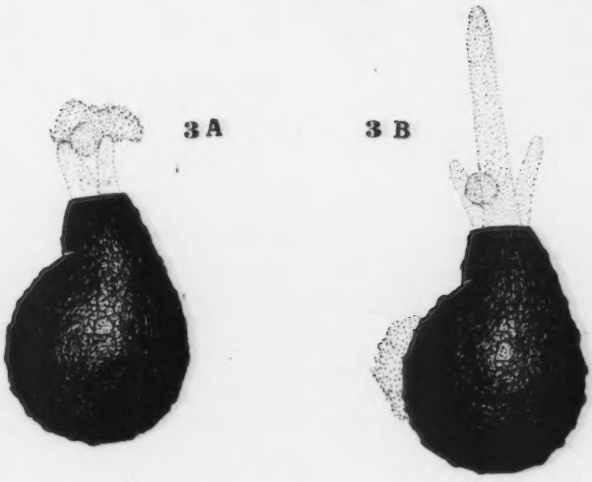
PLATE II.

FIG. 3. *Difflugia spiralis.* 3–*A.* Specimen placed in contact with a dead and a nearly dead fragment of its own ectoplasm. The dead fragment was at once rejected, the animal passing it (clinging along upper side of shell at 3-*B*) and dragging the nearly dead (spherical) fragment with it 3-*B*. In time both fragments were rejected.

Fig. 4. *Difflugia pyriformis.* Fragment *a* was whipped off by the sudden retraction of the pseudopod *A*. This fragment lay, of course, in position indicated by end of pseudopod *A*. For graphic reasons it and *b*, *c*, *d*, had to be placed below. While fragment *a* lay in this position, it lost its refractive axial rod as in *b;* put out a pseudopod to right as in *c;* assumed contour as in *d*. Next it travelled as an ameboid body to positions *e*, *f*, *g*. In the meantime pseudopod *A* had not reappeared and pseudopod *B* was thrown out behind and beyond *g*. When this met *g*, the currents in *g* were reversed and the body of *g* fused with the walls of the cup that *B* had formed about it, as shown at g'; as a result *g* flowed into *B* without leaving any local enlargement of *B*, or being seen as a discrete body within *B*.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3 B

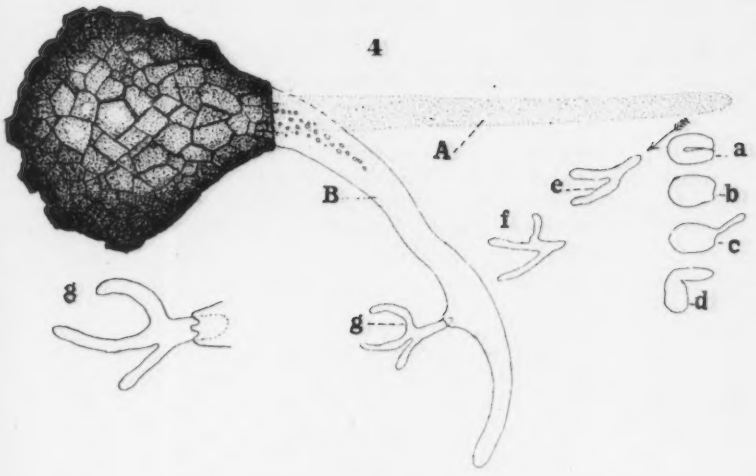

4
A
B
a
b
c
d
e
f
g
g

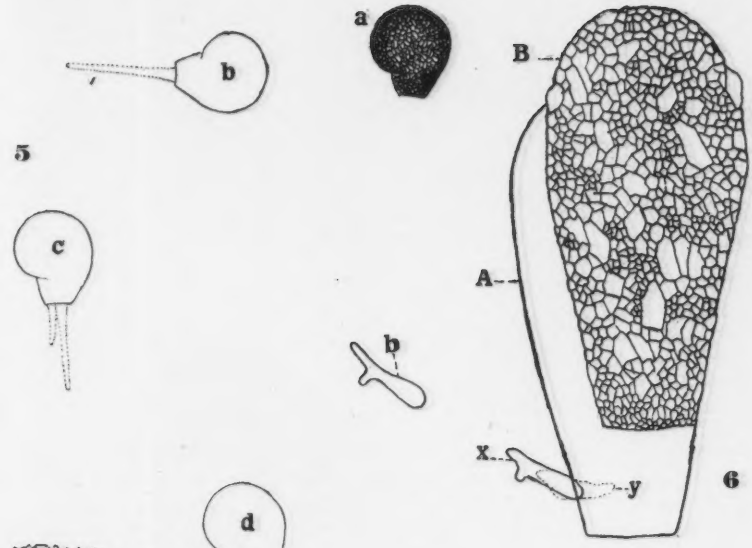
PLATE III.

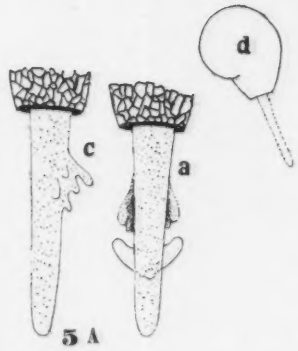
FIG. 5. *Difflugia spiralis.* Fragment cut from *a* and dragged to position 1, 1500μ distant, where the fragment had a rod-like contour. Until cell-body had moved through spiral curve along *b*, *c*, and to *d*, the fragment moved about aimlessly through positions 2, 3, 4, 5, 6, 7, 8, (9 the solid black outline not indicated by a numeral). The fragment and cell-body *d* now travelled towards each other, the former along path 10, 11, 12, 13, 14, and 15, the latter coming to position *e* where the two masses of protoplasm met. × 100.

FIG. 5–A. *a* shows fragment, left at 15 in Fig. 5, now travelling down along pseudopod towards a cup that had been formed; *b* union of fragment and cup just completed; *c* and *d* show the details of the end of this process of restitution. × 250.

FIG. 6. *Difflugia pyriformis.* Fragment *b* had moved from position *b* to *x* towards mouth of shell *A*. Cell-body was now moved to position *B* without disturbing either position or contour of *x*. Immediately after *A* was shifted to *B*, *x* changed its course and travelled to *y*. Here, unfortunately, the water had evaporated and in adding more water the fragment was lost. × 250.

FIG. 7. *Difflugia spiralis.* Two ectoplasmic fragments taken from an individual and placed side by side as at *m*. Here both are shown as living ameboid bodies. At *n* they are rounding up indicating the approach of death. While at *o* each fragment has become almost spherical and shows no signs of life. × 250.


a
b
5
B
A
c
b
x
y
6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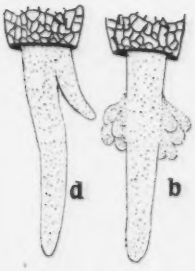
d
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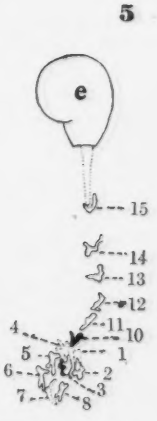
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1
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2
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3
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8

7
o
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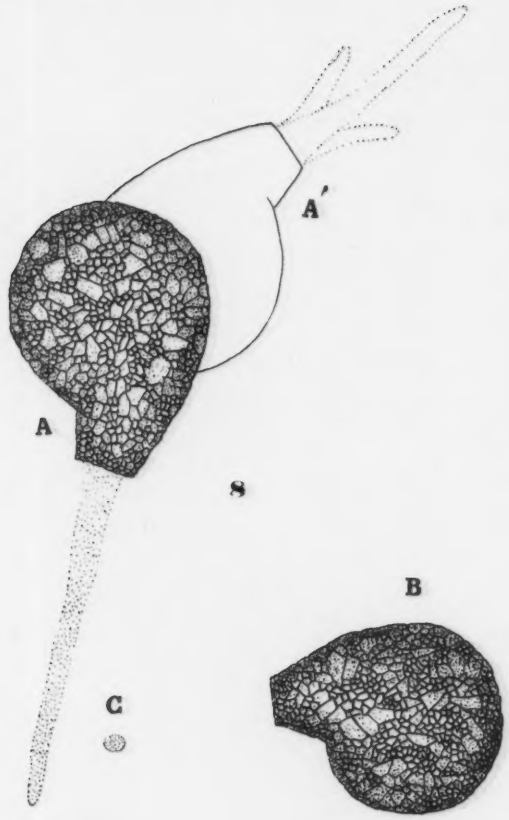
m

PLATE IV.

FIG. 8. *Difflugia spiralis.* At 11:29 A.M. specimen *A* lay as indicated in figure. Four minutes earlier a fragment had been taken from a pseudopod of this animal *A* and removed from the drop of water. Specimen *B* was brought to position indicated in figure. Fragment *C* was cut from *B* without disturbing *A* even so much as to cause it to retract its pseudopod. *B* was then removed. *A* travelled to position *A'* and moved out of field. It was then brought back to less than half a shell's length from fragment *C*, but though one of its pseudopods passed directly over *C*, it did not react to fragment *C*, nor did this fragment of *B*'s ectoplasm react towards the incomplete cell *A*. × 250.


A'
A
S
B
C